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Award Number: W81XWH-11-1-0541

TITLE: Pro-lipogenic action of lysophosphatidic acid in ovarian cancer

PRINCIPAL INVESTIGATOR: Xianjun Fang

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23219-1441

REPORT DATE: July 2012

TYPE OF REPORT: Revised Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2012	Revised Annual	1 July 2011-30 June 2012

4. TITLE AND SUBTITLE
Pro-lipogenic action of lysophosphatidic acid in ovarian cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0541

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Xianjun Fang

E-Mail: xfang@vcu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Virginia Commonwealth University

Richmond, VA 23219-1441

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The objective of the project is to determine the role of endogenous lysophosphatidic acid (LPA) in lipogenesis and metabolic abnormalities of ovarian cancer cells. During the first year of the funding support, we have pursued the specific aims and subaims as originally planned. The major discoveries include identification of LPA as a primary serum constituent to promote lipogenesis, role of iPLA2b in cell growth and potentially lipogenesis, downregulation of AMP:ATP ratio by LPA to inactivate AMPK, involvement of exogenous fatty acids via b oxidation to enhance LPA-induced cell growth, and LPA activation of the cholesterol rate-limiting synthesizing enzyme HMG-CoA reductase and cholesterol synthesis in ovarian cancer cells. Some of these results have been published in our recent paper (Journal of Biological Chemistry, 287: 24990–25000, 2012). The details of the unpublished results were presented and discussed in this report under each of the specific aims. We have also developed necessary reagents or learnt important techniques for the rest of the tasks. We expect to accomplish the project objectives within the 2nd year of the support with no major difficulty or change in research directions.

15. SUBJECT TERMS
LPA, lipogenesis, metabolic abnormality, iPLA2, ovarian cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

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Annual Progress Report for W81XWH-11-1-0541, Year 1

Introduction

Heightened *de novo* lipogenesis is a hallmark of human malignancies including ovarian cancer. The current ovarian cancer pilot research project titled “Pro-lipogenic action of lysophosphatidic acid in ovarian cancer” is to determine the role of endogenous lysophosphatidic acid (LPA) in lipogenesis and metabolic abnormalities of ovarian cancer cells. The scopes of research are Task 1: To define the role of endogenous LPA in regulation of lipogenesis in ovarian cancer cells; and Task 2: To determine the contribution of LPA-driven lipogenesis to metabolic abnormalities of ovarian cancer cells We have pursued these specific tasks as detailed below.

Body of Research Report

Specific Aims:

Aim 1. To define the role of endogenous LPA in regulation of lipogenesis in ovarian cancer cells

1.1 Examination of whether LPA in serum and LPA-inducing agents are sufficient to induce lipogenesis in ovarian cancer cells
We have examined whether serum stimulates *de novo* lipid synthesis in ovarian cancer cells. Serum strongly induced lipogenesis in OVCA-432 and Caov-3 cells. We have identified the LPA2 receptor as the primary receptor responsible for the lipogenic action of LPA as detailed in Fig. 5 of the attached manuscript. Knockdown of LPA2 expression not only inhibited LPA-induced lipogenesis (see the manuscript Fig. 5) but also significantly attenuated serum-driven lipogenesis (Fig. 1), suggesting that LPA is a major constituent of serum to promote lipogenesis in cancer cells. We are currently testing the effects of several LPA-inducing agents such as TPA and EGF (1).

1.2 Assessment of the effects of manipulating LPA-producing enzyme autotaxin (2) on activation of lipogenic pathways and *de novo* lipid synthesis in ovarian cancer cells
Two approaches have been utilized to address the role of autotaxin in activation of lipogenic pathways and *de novo* lipid synthesis in ovarian cancer cells. First, autotaxin was overexpressed in ovarian cancer cell lines. High levels of autotaxin protein were detectable in stable clones (data not shown). Second, we used autotaxin recombinant protein that we prepared previously. The recombinant protein was made in mammalian cells and of sufficient purity and activity as we described (3). We are currently testing appropriate doses and kinetics for further analysis in ovarian cancer cell lines.

1.3 Assessment of the effects on lipogenic enzymes and lipogenesis of pharmacological and molecular inhibition of iPLA2, another enzyme involved in LPA production in ovarian cancer cells iPLA2b is a potential enzyme involved in generation of endogenous LPA (4, 5). We generated stable clones to overexpress dominant negative form of iPLA2b (Fig. 2). Preliminary data indicate that ovarian cancer cell clones expressing the dominant negative iPLA2b were activity and growth inhibited (Fig. 2). However, it remains to be determined in the next few months if this is associated with inhibition of lipogenesis.

Aim 2. To determine the contribution of LPA-driven lipogenesis to metabolic abnormalities of ovarian cancer cells:

2.1 Analysis of the effects of LPA and LPA production on mitochondrial respiration in ovarian cancer cells
To this end, we proposed to analyze both cellular ATP levels and mitochondrial ATP production/oxygen consumption in ovarian cancer cells. This task relies on the development of the techniques. We have so far focused on the first part to analyze the effect of LPA on the total cellular ATP and AMP/ATP ratio, which could provide explanation on how LPA inactivates AMPK and activates ACC in ovarian cancer cells

(see the attached manuscript Fig. 3). We initially tried the luciferin/luciferase method to measure ATP. Although straightforward and reportedly to be quantitative (6), we found that the approach was largely qualitative with huge variations from experiment to experiment (data not shown). We thus switched to an HPLC-based assay which was more accurate and reproducible. As shown in Fig. 3, treatment of ovarian cancer cell line with LPA increased ATP production and decreased AMP/ATP ratio, a prominent regulator of the AMPK activity in mammalian cells.

2.2 Elucidation of the role of LPA and LPA production in regulation of lipid catabolic enzymes including monoacylglycerol lipase (MAGL) (7) and fatty acid beta oxidation (8) When they were treated with LPA to stimulate their proliferation, ovarian cancer cells were incubated with serum and lipid-free medium. An interesting question is whether the pro-lipogenic action of LPA is due to the lack of lipids in the extracellular environment. In other words, if provided with fatty acids, are ovarian cancer cells still stimulated by LPA to undergo active lipogenesis? To answer this, we have examined the effects of LPA in the presence of extracellular palmitate. Palmitate only slightly reduced LPA-dependent lipogenesis. The result has been published in the attached manuscript (Fig. 4A). In addition, we have also examined whether supply of fatty acids could potentiate LPA-induced cell growth. The data shown in Fig. 4 indicate that the presence of palmitate enhanced the growth-promoting activity of LPA in ovarian cancer cells. Since environmental fatty acids do not influence fatty acid synthesis, its effect on cell growth is most likely mediated by its catabolism through b-oxidation. We will further confirm this by examining b oxidation directly and the enzymes involved in fatty acid release.

2.3 Determination of the effects of LPA signaling on cholesterol synthesis and structures and functions of lipid rafts
We have examined the effects of LPA on the rate-limiting enzyme of cholesterol synthesis HMG-CoA reductase and on the cellular cholesterol levels. LPA stimulates HMG-CoA expression in ovarian cancer cells. The result is now published in Fig. 2B of the attached manuscript. LPA also stimulated production of cellular cholesterol (data not shown), consistent with the activation of HMG-CoA expression by LPA. We are currently isolating detergent soluble and resistant fractions from control and LPA-treated cells to determine if LPA alters structure and functions of lipid rafts as well as localizations of lipid raft-associated proteins.

2.4 Metabolic profiling of alterations in membrane and cellular lipids modulated by LPA using mass spectrometry

This will be major task for the 2nd year of the project. One graduate student in the lab is currently receiving technical training in the VCU mass spectrometry core.

Key Research Accomplishments

- Identification of LPA as a primary endogenous factor to promote lipogenesis;
- Discovery of the role of iPLA2b in cell growth and potentially lipogenesis;
- Discovery of LPA downregulation of AMP:ATP ratio to inactivate AMPK;
- Linking of exogenous fatty acids via b oxidation to LPA-induced cell growth; and
- Demonstration of LPA activation of HMG-CoA reductase, the cholesterol rate-limiting synthesizing enzyme and cholesterol synthesis in ovarian cancer cells.

Reportable Outcomes

Manuscript published:
Mukherjee A, Wu J, Barbour S and Fang X. Lysophosphatidic acid activates lipogenic pathways and de novo lipid synthesis in ovarian cancer cells. J Biol. Chem. 2012 287:24990-5000. PMID: 22665482

Abstracts:
Mukherjee A, Wu J, Barbour S, and Fang X. Lysophophatidic acid is a novel regulator of de novo lipogenesis in ovarian cancer. AACR special conference: Metabolism and Cancer. October 16-19, 2011, Baltimore, MD

Conclusions

During the first year of the funding support, we have pursued the specific aims and subaims as planned. The major results are summarized in key research accomplishments. Some of these results have been published in our recent paper attached. We have also developed necessary reagents or learnt important techniques in preparation for the remainder of the project as detailed under each specific aims. We expect to accomplish the project objectives within the 2nd year of the support with no major difficulty or change in research directions.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 30, pp. 24990–25000, July 20, 2012

Lysophosphatidic Acid Activates Lipogenic Pathways and *de Novo* Lipid Synthesis in Ovarian Cancer Cells*

Received for publication, January 9, 2012, and in revised form, May 31, 2012 Published, JBC Papers in Press, June 3, 2012, DOI 10.1074/jbc.M112.340083

Abir Mukherjee, Jinhua Wu, Suzanne Barbour, and Xianjun Fang[1]

From the Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, School of Medicine, Richmond, Virginia 23298

Background: Mechanisms underlying the lipogenic phenotype of cancer cells are poorly understood.
Results: Lysophosphatidic acid (LPA) via its receptor LPA_2 activates lipogenic pathways and *de novo* lipid synthesis in ovarian cancer cells.
Conclusion: LPA is causally linked to the aberrant lipogenesis in cancer.
Significance: This study offers a new strategy to inhibit lipid anabolism in a cancer cell-specific manner.

One of the most common molecular changes in cancer is the increased endogenous lipid synthesis, mediated primarily by overexpression and/or hyperactivity of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). The changes in these key lipogenic enzymes are critical for the development and maintenance of the malignant phenotype. Previous efforts to control oncogenic lipogenesis have been focused on pharmacological inhibitors of FAS and ACC. Although they show anti-tumor effects in culture and in mouse models, these inhibitors are nonselective blockers of lipid synthesis in both normal and cancer cells. To target lipid anabolism in tumor cells specifically, it is important to identify the mechanism governing hyperactive lipogenesis in malignant cells. In this study, we demonstrate that lysophosphatidic acid (LPA), a growth factor-like mediator present at high levels in ascites of ovarian cancer patients, regulates the sterol regulatory element binding protein-FAS and AMP-activated protein kinase-ACC pathways in ovarian cancer cells but not in normal or immortalized ovarian epithelial cells. Activation of these lipogenic pathways is linked to increased *de novo* lipid synthesis. The pro-lipogenic action of LPA is mediated through LPA_2, an LPA receptor subtype overexpressed in ovarian cancer and other malignancies. Downstream of LPA_2, the $G_{12/13}$ and G_q signaling cascades mediate LPA-dependent sterol regulatory element-binding protein activation and AMP-activated protein kinase inhibition, respectively. Moreover, inhibition of *de novo* lipid synthesis dramatically attenuated LPA-induced cell proliferation. These results demonstrate that LPA signaling is causally linked to the hyperactive lipogenesis in ovarian cancer cells, which can be exploited for development of new anti-cancer therapies.

One of the most common molecular changes in tumor cells is the heightened rate of *de novo* lipid synthesis compared with their normal counterparts. The aberrant lipogenesis in cancer cells is mediated by increased expression and activity of key lipogenic enzymes, primarily fatty acid synthase (FAS)[2] and acetyl-CoA carboxylase (ACC). Interestingly, the alterations in these key lipogenic enzymes are critical for the development and maintenance of the malignant phenotype (1). It occurs at early stages of tumorigenesis and becomes more pronounced in advanced cancers (1, 2). Overexpression of FAS correlates with poor prognosis in several types of human malignancies, including ovarian cancer (3, 4). Furthermore, tumor cells depend heavily on or are "addicted" to *de novo* lipid synthesis to meet their energetic and biosynthetic needs, irrespective of the nutritional supplies in the circulation (1). Consistent with this, pharmaceutical inhibitors of FAS suppress tumor cell proliferation and survival and enhance cytotoxic killing by therapeutic agents (5–10). However, one barrier to cancer patient applications of these inhibitors is their nonselective suppression of fatty acid synthesis in both normal and malignant tissues, which could deteriorate weight loss, anorexia, fatigue, and other cancer-associated complications. To target lipid anabolism in tumors specifically, it is important to identify the mechanism for the hyperactive lipogenesis in cancer cells, which is, however, poorly understood.

Lysophosphatidic acid (LPA), the simplest phospholipid, has long been known as a mediator of oncogenesis (11). LPA is present at high levels in ascites of ovarian cancer patients and other malignant effusions (11–13). LPA is a ligand of at least six G protein-coupled receptors (14). The LPA_1/Edg2, LPA_2/Edg4, and LPA_3/Edg7 receptors are members of the endothelial differentiation gene (Edg) family, sharing 46–50% amino acid sequence identity (14). GPR23/P2Y9/LPA_4 of the purinergic receptor family, and the related GPR92/LPA_5 and P2Y5/LPA_6 have been identified as additional LPA receptors, which are structurally distant from the LPA_{1-3} receptors (14, 15). The Edg LPA receptors, in particular LPA_2, is overexpressed in many types of human malignancies, including ovarian cancer (11, 16). Strong evidence implicates LPA_2 in the pathogenesis of ovar-

* This work was supported, in whole or in part, by National Institutes of Health Grants 2R01CA102196 and R21CA161478 from NCI (to X. F.). This work was also supported by Department of Defense Ovarian Cancer Research Program Grant W81XWH-11-1-0541 (to X. F.) and The Jeffress Memorial Fund award (to X. F.).

[1] To whom correspondence should be addressed. Tel.: 804-8280787; Fax: 804-828-1473; E-mail: xfang@vcu.edu.

[2] The abbreviations used are: FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; LPA, lysophosphatidic acid; AMPK, AMP-activated kinase; SREBP, sterol regulatory element-binding protein; qPCR, quantitative PCR; TAG, triacylglycerol.

ian, breast, and intestine tumors (16–18), although the exact oncogenic processes involved remain elusive.

In this study, we observed that LPA stimulated proteolytic activation of two isoforms of the sterol regulatory element-binding proteins (SREBPs), transcription factors involved in regulation of FAS and other lipogenic enzymes for biosynthesis of fatty acid and cholesterol. In addition, LPA induces dephosphorylation of AMPKα at Thr-172 and concomitant dephosphorylation of ACC at Ser-79. The dephosphorylation of ACC at Ser-79 is associated with activation of the enzyme (19). These LPA-induced changes in the lipogenic enzymes occurred hours after exposure to LPA, and the effects were sustained for many hours. Consistent with LPA activating these lipogenic pathways, LPA increased *de novo* lipid synthesis. We identified LPA_2, the receptor subtype overexpressed in ovarian cancer and other human malignancies, as the key receptor responsible for delivery of the lipogenic effect of LPA. The intracellular $G_{12/13}$-Rho signaling cascade is critical for LPA activation of the SREBP, whereas G_q-PLC is involved in LPA-mediated dephosphorylation and inhibition of AMPK. These findings reveal a novel mode of the cancer cell-specific regulation of lipogenesis by an intercellular factor present in the circulation and tumor microenvironments.

EXPERIMENTAL PROCEDURES

Reagents—LPA (1-oleolyl, 18:1) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, LPA was dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA) purchased from Roche Applied Science. Acetic acid (1-^{14}C) was obtained from Moravek Biochemicals (Brea, CA). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). The transfection reagent Dharmafect 1 was obtained from Dharmacon, Inc. (Lafayette, CO), and TransIT-TKO was obtained from Mirus Bio (Madison, WI). Luciferase assay reagents were obtained from Promega (Madison, WI). Anti-SREBP-1 and anti-SREBP-2 antibodies were obtained from BD Biosciences. Anti-phospho-AMPKα (Thr-172), anti-AMPKα, anti-phospho-ACC (Ser-79), anti-ACC, and anti-FAS antibodies were obtained from Cell Signaling (Danvers, MA). Anti-tubulin antibody was obtained from EMD4Biosciences (Gibbstown, NJ). BODIPY 493/503 and cell culture reagents were purchased from Invitrogen. The TaqMan Universal PCR Master Mix and qPCR probes for LPA_1, LPA_2, LPA_3, 3-hydroxy-3-methylglutaryl-CoA (HGM-CoA) reductase, and GAPDH were obtained from Applied Biosystems (Carlsbad, CA). Calpain I inhibitor, water-soluble cholesterol, the FAS inhibitor C75, the ACC inhibitor TOFA, and sodium palmitate were purchased from Sigma.

Cell Culture—The sources of ovarian cancer cell lines used in the study were described previously (20). These cells were cultured in RPMI medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. IOSE-29 was originally obtained from Dr. N. Auersperg (University of British Columbia, Canada) and cultured as described previously (21).

siRNA, Plasmids, and Transfection—The siRNA oligos for LPA_1, LPA_2, LPA_3, and FAS were obtained from Applied Biosystems. These siRNAs were transfected into cells using Dharmafect 1 following the manufacturer's protocol. In brief, cells were plated in 6-well plates to reach 50–60% confluence before transfection. Cells were then transfected with target-specific siRNA or nontargeting control siRNA (150 pM) with Dharmafect 1 (4 μl) for 12–16 h. Approximately 48 h post-transfection, the cells were serum-starved overnight before LPA treatment. Lentiviruses carrying short hairpin RNA (shRNA) for LPA_{1-3} receptors were kind gifts from Dr. S. Huang (Medical College of Georgia) (22). The expression vector pcDNA3 expressing the dominant negative form of G_i was provided by Dr. P. Hylemon (Virginia Commonwealth University) (23, 24). The G_q and G_{12} cDNAs were provided by Dr. R. D. Ye (University of Illinois at Chicago). The dominant negative mutants of G_q (G208A) and G_{12} (G228A) (25–27) in pcDNA3 were made using the QuikChange XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA). The plasmids and the vectors expressing N19Rho and botulinum toxin C3 were described previously (28, 29). These plasmids were transfected into ovarian cancer cell lines using Lipofectamine LTX Plus (Invitrogen) following the manufacturer's instruction.

Luciferase Assays—The SREBP-responsive luciferase reporter vector (pGL2–3×SREBP-TK-Luc) was generated by cloning three repeats of the SREBP consensus sequence (AAAATCACCCCACTGCAAACTCCTCCCCCTGC) (30, 31) into the NheI and HindIII sites in front of the herpes simplex virus thymidine kinase gene promoter (−35 to +50) in the pGL2-TK-Luc vector (32). Ovarian cancer cell lines were transfected with the luciferase vector using TransIT-TKO according to the manufacturer's protocol. About 48 h after transfection, the cells were starved overnight and treated with LPA or vehicle (BSA) for 12 h. Cell extracts were prepared and assayed for luciferase activity using the luciferase assay kits from Promega.

Western Blotting—Cells were lysed as described previously (33). Total cellular proteins were resolved by SDS-PAGE, transferred to immunoblot membrane (polyvinylidene difluoride) (Bio-Rad), and immunoblotted with antibodies following the protocols of the manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit from Amersham Biosciences.

Quantitative PCR (qPCR)—Total cellular RNA was isolated from cultured cells using TRIzol (Invitrogen). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). The relative levels of LPA_1, LPA_2, LPA_3, HMG-CoA reductase, and GAPDH were determined by qPCR using gene-specific probes, the TaqMan Universal PCR master mix, and the Applied Biosystems 7900HT real time PCR system.

Measurement of de Novo Lipid Synthesis—Cells were grown in 6-well plates and serum-starved prior to treatment with LPA or vehicle for 24 h. The cells were labeled with [^{14}C]acetic acid (5 μCi/ml) for the last 6 h of incubation. The cells were then washed twice with PBS and lysed with lysis buffer (25 mM HEPES, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.2 mM EDTA, 0.5% sodium deoxycholate, 20 mM glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Lipids were extracted using a chloroform/methanol solution (2:1). Phase separation was achieved by centrifugation at 3200 × *g* for 10 min. The organic phase was extracted and dried with a speed vacuum. Lipids were dissolved

in Ultima Gold Mixture (PerkinElmer Life Sciences) and counted using Beckman LS 6500 scintillation counter. Each measurement was performed in triplicate and normalized to cell numbers.

Lipid Staining—Cells were grown and serum-starved prior to treatment with LPA or vehicle for 24 h. Cells were then stained with BODIPY 493/503 at a final concentration of 0.5 μg/ml in PBS at 37 °C for 30 min, followed by counter-staining with Hoechst (10 μg/ml) for 15 min. Cells were then fixed with 2% paraformaldehyde and visualized with fluorescence microscopy.

Quantification of Triacylglycerols (TAG) and Phospholipids—TAG and phospholipids were extracted and quantified with the EnzyChrom triglyceride assay kit and the EnzyChrom phospholipid assay kit (BioAssay Systems, Hayward, CA), respectively, according to the manufacturer.

Statistics—All numerical data were presented as means ± S.D. The statistical significance of differences was analyzed using Student's *t* test, where $p < 0.05$ was considered statistically significant. In all figures, the statistical significances were indicated with an asterisk if $p < 0.05$ or two asterisks if $p < 0.01$.

RESULTS

LPA Induces Proteolytic Cleavage and Activation of SREBP in a Cholesterol-sensitive Manner—The hyperactive lipogenesis is a hallmark of tumor cells (1, 34). To identify pathophysiological mechanisms driving the lipogenic program in cancer cells, we examined the potential role of LPA, an endogenous regulator of many cellular functions in ovarian cancer and other human malignancies. We first assessed whether LPA was capable of activating the SREBP transcription factors that play crucial roles in regulating expression of lipogenic enzymes. Treatment of Caov-3, OVCA-432, and other ovarian cancer cell lines, including OVCAR-3, with LPA induced cleavage of the precursor forms of SREBP-1 and SREBP-2 in a time-dependent manner (Fig. 1*A*). The cleaved mature forms of SREBP-1 and SREBP-2 were detectable at 4 h and peaked at 12 h post-LPA treatment. In contrast to the ovarian cancer cell lines, LPA failed to activate SREBP-1 or SREBP-2 in the immortalized ovarian surface epithelial cell line IOSE-29 (Fig. 1*A*) or normal ovarian epithelial cells (data not shown), suggesting a cancer cell-specific mechanism for SREBP activation by LPA in ovarian cancer cells.

In physiological conditions, SREBP-1 and SREBP-2 are regulated by the intracellular sterol content. In their precursor forms, SREBPs are attached to the endoplasmic reticulum. Specific signaling cues such as reduced cholesterol levels trigger SREBP cleavage-activating protein (SCAP)-mediated transport of SREBP from endoplasmic reticulum to Golgi, where they are cleaved by proteases S1P and S2P to release the mature/active form (35). At high sterol concentrations, the SREBP-SCAP complex is retained in the endoplasmic reticulum due to increased binding to INSIG proteins (36). To determine whether LPA activation of SREBP could bypass cholesterol regulation, we preloaded Caov-3 and OVCA-432 cells with cholesterol (10 μg/ml) complexed with 0.1% fraction V fatty acid-free BSA in PBS, and then assessed activation of SREBP-1 in response to LPA. As shown in Fig. 1*B*, cholesterol treatment reduced both basal and LPA-induced active SREBP-1 levels, indicating that activation of SREBP by LPA remains sensitive to the cholesterol availability.

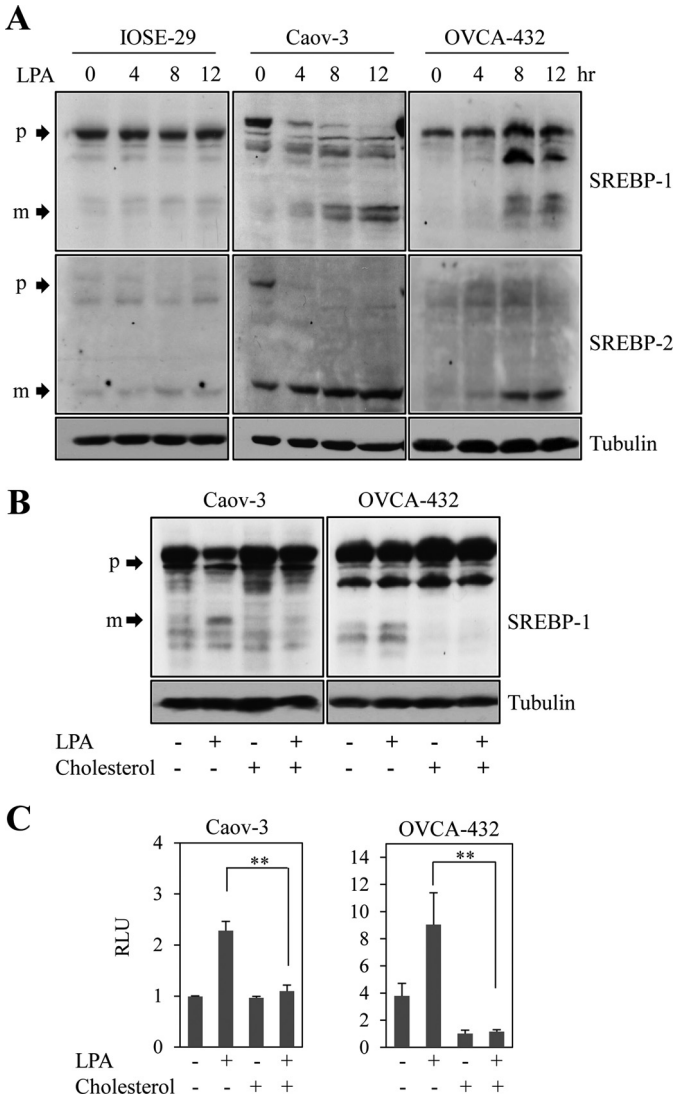

FIGURE 1. **LPA activates SREBP in ovarian cancer cells.** Ovarian cancer cell lines and IOSE-29 cells were treated with LPA (10 μM) for indicated periods of time. The calpain inhibitor I (25 μg/ml) was added to cells for the last 2 h. Expression of SREBP-1 and SREBP-2 was analyzed by immunoblotting with antibodies that recognize both precursor (*p*) and active/mature (*m*) forms of SREBP-1 and SREBP-2 (*A*). *B*, Caov-3 and OVCA-432 cells were preloaded with or without cholesterol (10 μg/ml). The cells were treated with LPA and analyzed for expression of precursor and mature forms of SREBP as in *A*. *C*, Caov-3 and OVCA-432 cells were transfected with pGL2–3×SREBP-TK-Luc and loaded with or without cholesterol before stimulation with LPA (10 μM) for 12 h. The luciferase activity in cell extracts was determined as described under "Experimental Procedures," and the results are presented as relative luciferase units (*RLU*).

To determine whether LPA-induced SREBP cleavage is sufficient to activate SREBP transcriptional activity, Caov-3 and OVCA-432 cells were transfected with the SREBP-responsive reporter pGL2–3×SREBP-TK-Luc. As shown in Fig. 1*C*, treatment of transfected cells with LPA significantly enhanced luciferase activity in these cells. Similar to the SREBP cleavage, SREBP-dependent luciferase activity was also sensitive to cholesterol treatment (Fig. 1*C*).

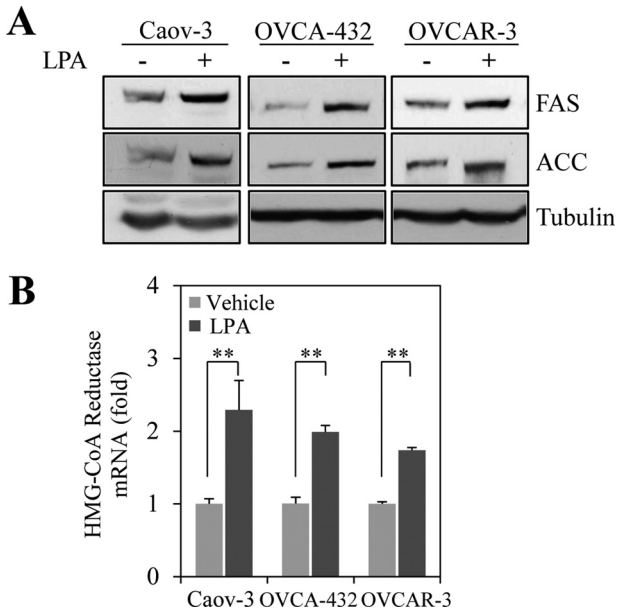

FIGURE 2. **LPA induces expression of the SREBP target genes FAS, ACC, and HMG-CoA reductase.** Caov-3, OVCA-432, and OVCAR-3 cells were treated with or without LPA (10 μM) for 16 h prior to immunoblotting analysis of FAS and ACC (*A*). Total cellular RNA was isolated from parallel samples and subjected to qPCR analysis of expression of HMG-CoA reductase mRNA (*B*). The results are presented as fold increase relative to the value in the vehicle-treated cells (defined as *1*).

LPA Induces Expression of SREBP Target Genes FAS, ACC, and HMG-CoA Reductase—To substantiate the biological significance of SREBP activation by LPA, we monitored expression levels of FAS, ACC, and HMG-CoA reductase. These are well known targets of SREBP-1 and SREBP-2 involved in biosynthesis of fatty acid and cholesterol. Treatment of Caov-3, OVCA-432, and OVCAR-3 cells with LPA increased expression levels of FAS and ACC proteins as shown in Fig. 2*A*. The mRNA levels of these key enzymes for fatty acid synthesis (data not shown) and the rate-limiting enzyme for cholesterol synthesis HMG-CoA reductase were also significantly increased by treatment of ovarian cancer cell lines with LPA (Fig. 2*B*), providing evidence that activation of SREBP-1 and SREBP-2 by LPA is sufficient to increase expression of key endogenous lipogenic enzymes in ovarian cancer cells.

LPA Induces Dephosphorylation of AMPK and ACC—In addition to transcriptional up-regulation, the activity of ACC is inhibited by AMPK-mediated phosphorylation. AMPK, a highly conserved protein serine/threonine kinase, acts as an energy sensor and regulator of cellular metabolism, shutting down energy-consuming anabolic processes and activating energy-yielding catabolic processes (37). AMPK is activated through phosphorylation of Thr-172 within the activation domain of the α-subunit (38). To determine the effect of LPA on AMPK and its downstream target ACC, we analyzed the phosphorylation status of AMPKα at this residue as a surrogate of activation of the enzyme. Treatment of Caov-3 and OVCA-432 cells with LPA induced late onset and sustained dephosphorylation of AMPKα (Fig. 3). The decrease in AMPKα phosphorylation was detectable at 8 h and became prominent at 12 h. Consistent with a predominant role of AMPKα in phosphorylation of ACC, AMPKα dephosphorylation in LPA-

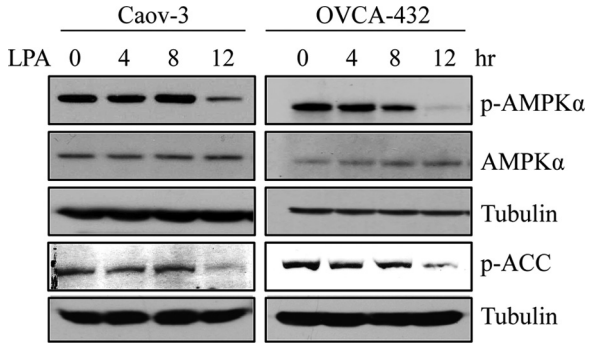

FIGURE 3. **LPA induces dephosphorylation of AMPKα and ACC.** Caov-3 and OVCA-432 cells were treated with or without LPA (10 μM) for the indicated periods of time. The cell lysates were analyzed with immunoblotting for phosphorylation status of AMPKα and ACC using their phospho-specific antibodies recognizing AMPKα phosphorylated at Thr-172 or ACC phosphorylated at Ser-79.

treated cells was accompanied by a decrease in ACC phosphorylation at Ser-79 (Fig. 3). Dephosphorylation of this site is known to enhance ACC enzymatic activity. The effects of LPA on dephosphorylation of AMPKα and ACC were not detected in IOSE-29 cells (data not shown). These results establish that LPA signaling is coupled to activation of ACC via inhibition of AMPK.

LPA Promotes de Novo Lipid Synthesis—Few studies have examined the role of exogenous factors in regulation of lipogenesis in cancer cells (5, 39). We examined whether LPA-induced activation of lipogenic enzymes is functionally sufficient to stimulate *de novo* lipid synthesis. The ovarian cancer cell lines Caov-3 and OVCA-432 and the immortalized IOSE-29 cells were treated with LPA or BSA as vehicle control and pulse-labeled with [^{14}C]acetic acid to monitor the amount of new lipid synthesis. As demonstrated in Fig. 4*A* (*left panel*), LPA treatment led to a significant increase in ^{14}C incorporation into the cellular lipid fractions, reflecting an increase in newly synthesized lipids in response to LPA. The lipogenic effect of LPA was specifically detected in multiple ovarian cancer cell lines but not in the nontransformed IOSE-29 cells, wherein LPA failed to induce SREBP activation or AMPK dephosphorylation. Because these cells were treated with LPA in serum-free medium lacking extracellular fatty acids, we wanted to determine whether the increase in lipogenesis in response to LPA is influenced by availability of extracellular lipids. As shown in Fig. 4*A* (*right panel*), exogenously supplemented palmitate slightly reduced LPA-driven lipogenesis. The reduction was, however, statistically insignificant, indicating that the lipogenic role of LPA is largely independent of availability of extracellular fatty acids. Consistent with the pro-lipogenic action of LPA, staining with the lipophilic dye BODIPY 493/503 revealed that LPA induced moderate increases in the intracellular contents of neutral lipids in Caov-3 and OVCA-432 cells but not in IOSE-29 cells (Fig. 4*B*). These results were further supported by the increases in both cellular TAG and phospholipids following LPA treatment (Fig. 4, *C* and *D*).

LPA$_2$ Is Major Receptor Subtype Responsible for Regulation of SREBP and AMPK—Caov-3, OVCA-432, and other ovarian cancer cell lines express the Edg LPA receptors LPA$_1$, LPA$_2$, and LPA$_3$ (Fig. 5*A*). The other non-Edg LPA receptors are

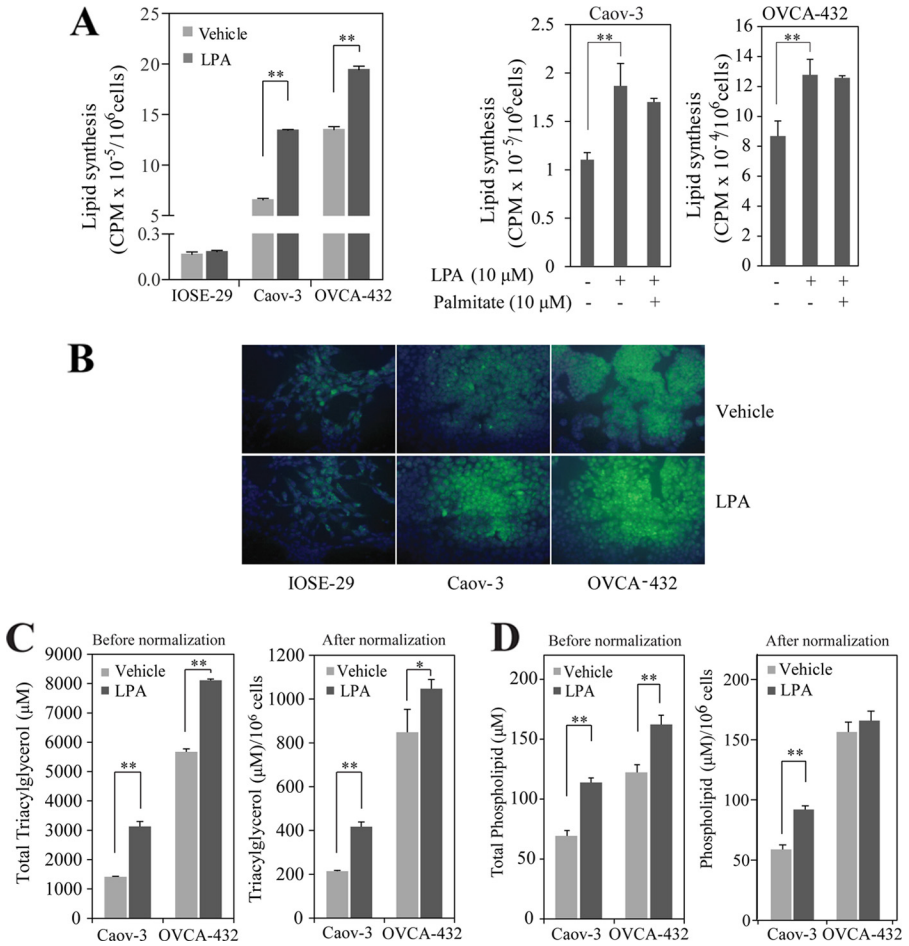

FIGURE 4. **LPA stimulates *de novo* lipid synthesis.** Caov-3, OVCA-432, and IOSE-29 cells were treated with LPA (10 μM) or BSA (vehicle) for 24 h. In the last 6 h of incubation, the cells were pulse-labeled with 5 μCi/ml of [^{14}C]acetic acid before lipid extraction as described under "Experimental Procedures." The incorporation of ^{14}C into lipid fractions was determined by scintillation counting. The results were presented as counts/min per 1×10^6 cells (*A*, *left panel*). Caov-3 and OVCA-432 cells were treated with LPA in serum-free medium supplemented with palmitate (10 μM) and BSA (0.01%). LPA-induced lipogenesis was measured as described above (*A, right panel*). *B*, the parallel cells in 6-well plates were stained with BODIPY 493/503 fluorescent dye (0.5 μg/ml) for 30 min, followed by staining with Hoechst (10 μg/ml) for 15 min to monitor lipid accumulation. Shown were fluorescence microscopic photographs of IOSE-29, Caov-3, and OVCA-432 cells treated with or without LPA (×80 magnification). Total TAG (*C*) and phospholipids (*D*) in control and LPA-treated Caov-3 and OVCA-432 cells were determined as described under "Experimental Procedures." The results are presented as amounts of lipids per well or normalized on cell numbers to represent amounts of lipids per million cells.

either absent or expressed inconsistently in ovarian cancer cells (40, 41). Thus, we focused on the potential role of LPA_{1-3} in the regulation of lipogenesis. We used siRNA to knock down expression of LPA_1, LPA_2, and LPA_3 in Caov-3 cells and examined SREBP activation and AMPKα dephosphorylation in response to LPA treatment. Interestingly, only knockdown of LPA_2 remarkably attenuated LPA-induced cleavage of SREBP-1, dephosphorylation of AMPKα at Thr-172 (Fig. 5*B*), as well as expression of FAS and ACC (Fig. 5*C*). There were little inhibitory effects on SREBP-1 activation, AMPKα dephosphorylation, and expression of FAS and ACC in conjunction with LPA_1 or LPA_3 knockdown. We encountered a technical difficulty in achieving efficient knockdown of LPA receptors with transient siRNA in OVCA-432 cells. However, similar results were obtained from OVCA-432 cells when LPA receptors were stably knocked down by lentivirus-transduced shRNA (Fig. 5, *B* and *C*). These results support a primary role of the LPA_2 receptor in LPA-dependent activation of SREBP-1 and inhibition of AMPKα. However, overexpression of LPA_2 in IOSE-29 cells was not sufficient to activate LPA-dependent induction of FAS and ACC (data not shown), suggesting that additional signaling player(s) present specifically in malignant cells is involved.

To verify this receptor subtype-specific regulation of lipogenesis, we examined the effect of LPA_2 knockdown on LPA-driven lipogenesis. The *de novo* lipid synthesis in LPA receptor knockdown and control cells was assessed as described earlier. The endogenous lipid synthesis induced by LPA was strongly attenuated by siRNA- or shRNA-mediated down-regulation of LPA_2 (Fig. 5*D*). In contrast, knockdown of LPA_3 (Fig. 5*D*) or LPA_1 (data not shown) did not inhibit LPA-induced lipid synthesis.

LPA_2 Signaling Bifurcates to Regulate SREBP-1 and AMPKα—We next examined the signaling effectors downstream of LPA_2 responsible for cleavage of SREBP-1 and dephosphorylation of AMPKα. The LPA_{1-3} receptors couple to G_i and G_q, whereas only LPA_1 and LPA_2 couple to $G_{12/13}$ (42). We transfected dominant negative forms of these G proteins into highly transfectable Caov-3 cells in an effort to screen for G proteins critical for LPA-dependent SREBP-1 cleavage and AMPKα dephosphory-

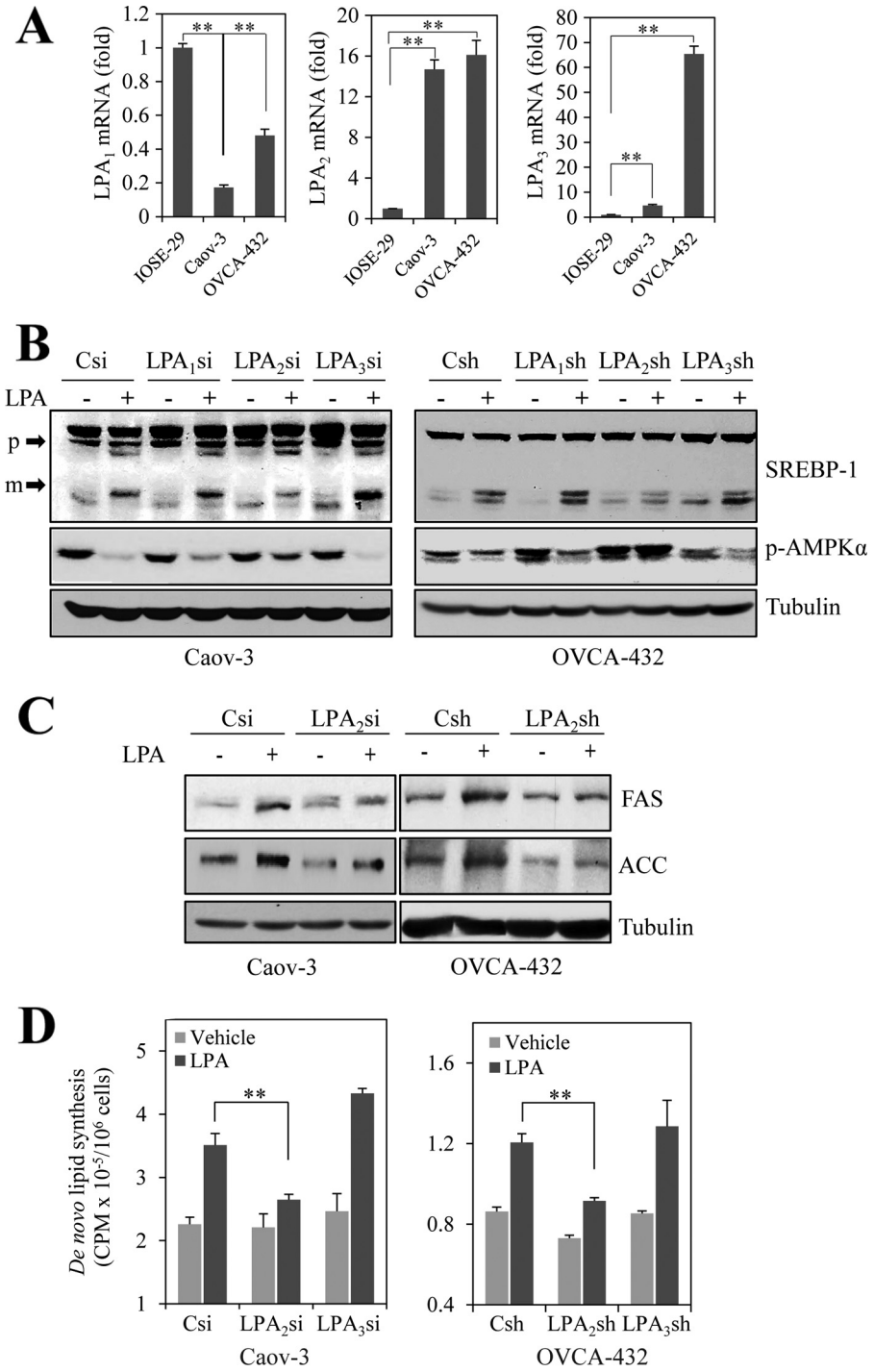

FIGURE 5. **LPA_2 mediates the lipogenic effect of LPA.** Expression of mRNAs of LPA_{1-3} receptors in IOSE-29, Caov-3, and OVCA-432 cells was determined by qPCR analysis as detailed under "Experimental Procedures" (*A*). The results were presented as fold difference relative to the mRNA levels of LPA receptors in IOSE-29 cells (defined as *1*). Caov-3 cells were transfected with siRNA for each LPA receptor (LPA_1si, LPA_2si, and LPA_3si) or with nontargeting control siRNA (*Csi*). Expression of each LPA receptor in OVCA-432 cells was down-regulated by lentivirus-transduced shRNA. The knockdown efficiencies for each LPA receptor in both cell lines range from 60 to 80% as determined by qPCR analysis (data not shown). The cells were stimulated with LPA (10 μM) for 12 h before immunoblotting analysis of SREBP-1 and phospho-AMPKα (*B*). *p*, precursor; *m*, active/mature. *C*, effects of LPA_2 knockdown on FAS and ACC induction in Caov-3 and OVCA-432 cells were examined by immunoblotting analysis. *D*, effects on lipid synthesis of siRNA or shRNA knockdown of LPA_1, LPA_2, or LPA_3 receptor in Caov-3 and OVCA-432 cells were measured as described in Fig. 4*A*.

lation. As shown in Fig. 6*A*, expression of the dominant negative G_{12} attenuated LPA-induced SREBP-1 cleavage but not LPA-induced dephosphorylation of AMPKα. In contrast, expression of dominant negative G_q inhibited AMPKα dephosphorylation but not SREBP-1 cleavage induced by LPA. Thus, different G protein cascades are implicated in the regulation of SREBP and AMPK by LPA. Because a prominent effector of $G_{12/13}$ is the Rho GTPase, we examined whether Rho is required for LPA activation of SREBP. As expected, expression of dominant negative Rho (N19Rho) or the botulinum toxin C3,

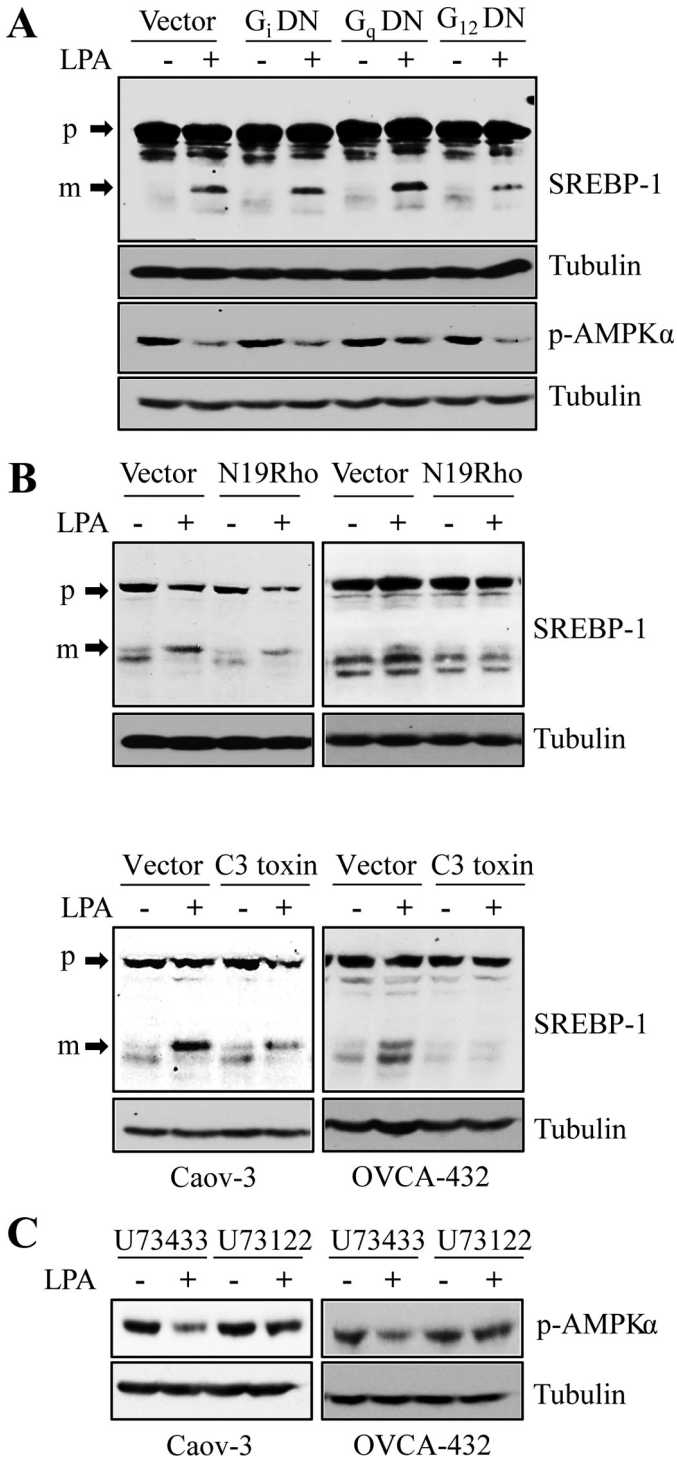

FIGURE 6. **LPA regulates SREBP and AMPK through different G protein cascades.** Caov-3 cells were transfected to express dominant negative forms of G_i, G_q, and G_{12} or the control vector. The transfected cells were treated with LPA (10 μM) for 12 h before immunoblotting analysis of SREBP-1 cleavage and AMPKα dephosphorylation (*A*). *p*, precursor; *m*, active/mature. *B*, dominant negative Rho (*N19Rho*) or C3 toxin expression vector was transfected into Caov-3 and OVCA-432 cells. The effects of N19Rho and C3 toxin on LPA-induced SREBP-1 cleavage were analyzed by immunoblotting. *C*, Caov-3 and OVCA-432 cells were treated with LPA in the presence of the PLC inhibitor U73122 or its inactive analog U73433 (10 μM). LPA-induced AMPKα dephosphorylation was analyzed by immunoblotting.

a specific inhibitor of Rho GTPase, suppressed LPA-induced cleavage of SREBP-1 (Fig. 6*B*) as compared with vector-transfected cells. The results demonstrate that LPA_2 promotes SREBP activation in a Rho-dependent pathway.

To elucidate the regulatory network leading to AMPK dephosphorylation, we used pharmacological inhibitors of signaling molecules downstream of G_q. As shown in Fig. 6*C*, the PLC inhibitor U73122 but not it's inactive analog U73433 blocked AMPKα dephosphorylation induced by LPA. The data support a G_q-PLC-dependent mechanism to control phosphorylation and activity of AMPKα in LPA-treated cells.

LPA-driven Cell Proliferation Requires LPA_2 and de Novo Lipid Synthesis—LPA is a mitogen that stimulates proliferation of ovarian cancer cells (43–46). To understand the biological significance of LPA-induced lipogenesis, we examined whether the pro-lipogenic activity of LPA contributes to LPA-driven proliferation of ovarian cancer cells. C75 and TOFA are well characterized specific inhibitors of FAS and ACC, respectively (47, 48). The presence of C75 dramatically decreased cell numbers of Caov-3 and OVCA-432 cells in serum-free medium supplemented with LPA as a growth factor (Fig. 7*A*), suggesting that the blockade of *de novo* lipogenesis could attenuate LPA-induced cell proliferation. Similar effects were observed in the presence of the ACC inhibitor TOFA (data not shown). At the concentrations we used, C75 and TOFA did not induce significant increases in apoptosis or appreciable decreases in cell viability (data not shown), suggesting that these inhibitors mainly targeted cell proliferation rather than cell survival. We also tested if exogenously added palmitate could reverse the effect of C75 on LPA-induced cell proliferation. At 10 μM, palmitate partially prevented the effect of C75 (Fig. 7*B*). This ability of palmitate, however, was not seen at 20 μM, suggesting possible cytotoxic effect of high concentrations of palmitate.

To obtain molecular evidence for involvement of FAS in LPA-induced cell proliferation, we used siRNA to knock down FAS expression in Caov-3 and OVCA-432 cells. Down-regulation of FAS expression indeed prevented proliferation of these cells induced by LPA (Fig. 7*C*). Finally, because LPA_2 is the key receptor subtype required for LPA activation of lipogenesis, we knocked down its expression to determine whether LPA_2 is an integral component of LPA-induced cell proliferation. As shown in Fig. 7*D*, following down-regulation of LPA_2, both cell lines exhibited significant decrease in growth rate when the cells were incubated in serum-free medium containing LPA. Thus LPA_2 and its associated lipogenesis-promoting activity are critical for LPA-induced cell proliferation.

DISCUSSION

The majority of the adult tissues depends on dietary fat to meet their nutritional needs. In contrast, cancer cells depend on *de novo* lipid synthesis for generation of fatty acids, irrespective of the available extracellular supplies. Malignant cells typically show a high rate of *de novo* fatty acid synthesis (49, 50). Intracellular fatty acids in rapidly dividing cancer cells not only supply energy through β-oxidation but more importantly serve as precursors for biosynthesis of membrane phospholipids, signaling lipids, and secondary messengers (51). The lipogenic phenotype of cancer cells has been primarily attributed to

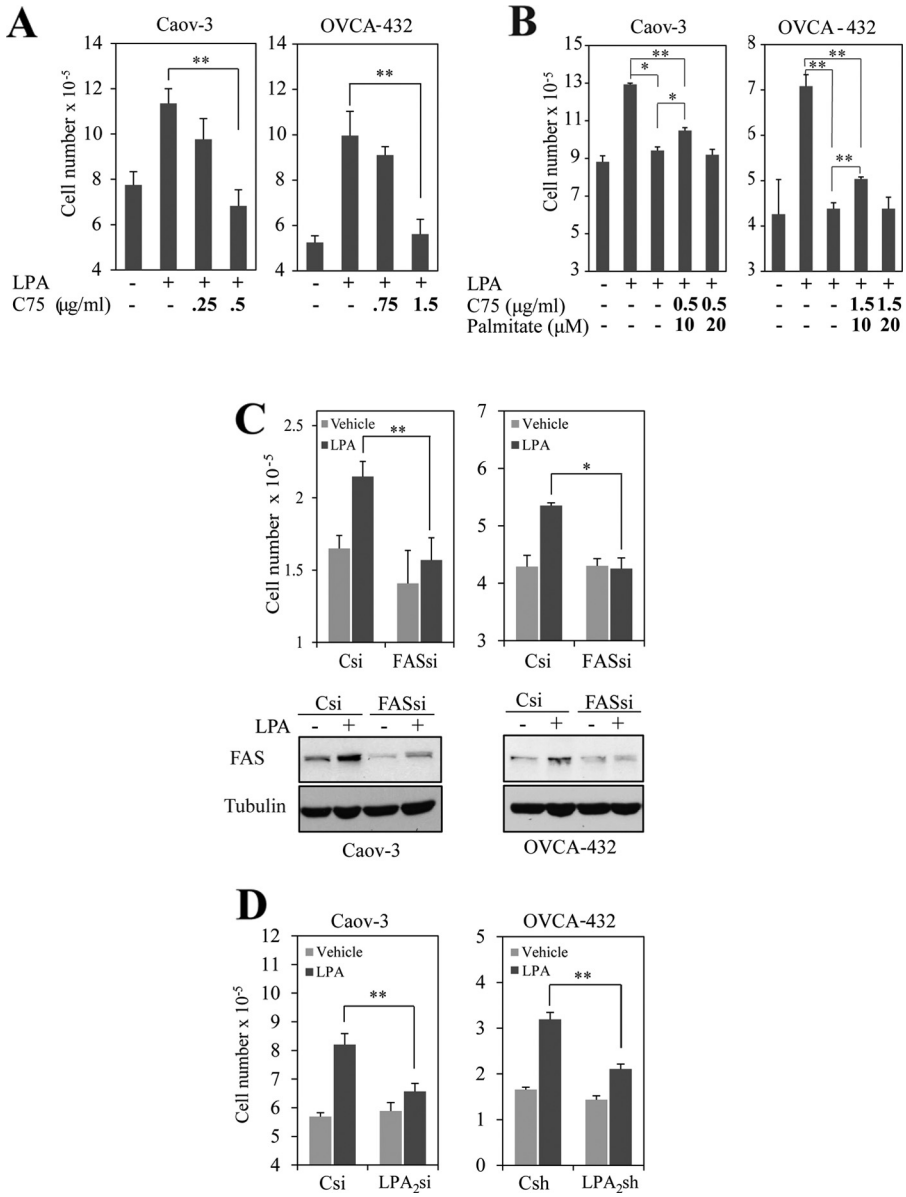

FIGURE 7. **LPA_2 and associated lipogenic activity are required for LPA-induced cell proliferation.** Caov-3 and OVCA-432 cells in 6-well plates were incubated for 48 h in serum-free medium supplemented with 10 μM LPA in the presence of indicated concentrations of the FAS inhibitor C75 (*A*). *B*, Caov-3 and OVCA-432 cells were incubated with LPA (10 μM) and C75 in the presence of the indicated concentrations of palmitate. BSA was kept at a final concentration of 0.01% for all treatments. *C* and *D*, expression of FAS (*C*) or LPA_2 (*D*) was down-regulated by siRNA knockdown in Caov-3 and OVCA-432 cells to examine LPA-induced cell proliferation after 48 h of incubation with 10 μM LPA. In all panels, cell numbers were quantitated with Coulter counter and presented as mean ± S.D. of triplicate assays, representative of three independent experiments.

increased expression or aberrant activity of the major lipogenic enzymes FAS and ACC. In particular, FAS, originally recognized as a tumor-specific antigen present in serum of cancer patients (34), is overexpressed in a variety of human malignancies. However, the cellular mechanisms by which lipogenic enzymes are up-regulated in cancer cells remain poorly understood except for a few studies suggesting that steroid hormones and Her family ligands could increase FAS expression via the PI3K or MAPK pathways (52–55).

In this study, we describe a novel LPA-mediated mechanism activating *de novo* lipogenesis in ovarian cancer cells. We demonstrated that treatment of ovarian cancer cell lines with LPA activates the SREBP-FAS and AMPK-ACC lipogenic cascades, culminating in increased *de novo* lipid synthesis. The lipogenic effect of LPA was specifically observed in cancer cells as LPA failed to induce *de novo* lipogenesis in nontransformed IOSE-29 cells. LPA has been long known as a mediator of ovarian cancer. It is present at high concentrations in tumor microenvironments such as ascites of ovarian cancer patients and other malignant effusions (12, 13). This study highlights the possibility that LPA is an etiological factor in tumor microenvironments to promote lipogenesis in ovarian cancer cells, although the effect of LPA in other cancer cells remains to be determined.

A significant finding of this study is the selective role of the LPA_2 receptor in LPA activation of the lipogenic pathways and LPA-driven lipogenesis. We and others have previously shown that LPA_2 and LPA_3 are overexpressed in significant fractions

of ovarian cancer and in most ovarian cancer cell lines (16, 46). LPA_1, which is expressed by both normal and malignant ovarian epithelial cells, is dispensable for the pro-lipogenic activity of LPA in ovarian cancer cells. It is somewhat surprising that in both Caov-3 and OVCA-432 cells, knockdown of LPA_3 slightly potentiated the lipogenic effect of LPA (Fig. 5*D*). The results imply that the crosstalk among co-expressed LPA receptors is important in the control of biological outcomes of LPA. The specific role of LPA_2 in the promotion of lipogenesis in tumor cells is consistent with the increased expression of this receptor in various malignancies (16, 56–58). Although LPA_1 and LPA_3 have also been reported to be up- or down-regulated in some cancers, overexpression of LPA_2 is most commonly seen in almost all cancer types examined (16, 56–58). There is also strong evidence from xenograft mouse models and transgenic mice that LPA_2 is more oncogenic compared with LPA_1 and LPA_3 (17, 59). The compelling evidence for the implication of LPA_2 as an oncogene stems from recent studies by Yun and co-workers (18, 60) who showed that LPA_2-deficient mice were more resistant to intestinal tumorigenesis induced by colitis or by ApcMin mutation. However, the molecular mechanisms for the oncogenic activity of LPA_2 are not well understood. Most previous studies have been focused on the ability of LPA_2 to stimulate expression of oncogenic protein factors, including IL-6, VEGF, HIF1α, c-Myc, cyclin D1, Krüppel-like factor 5, and Cox-2 (18, 32, 60–63). LPA_2 seems to be more potent than other LPA receptors in driving the transcriptional effects of LPA on these LPA target genes. This study links LPA_2 to the lipogenic phenotype of ovarian tumor cells. The role of LPA_2 in lipid metabolism provides a new avenue to explore the oncogenic role of LPA.

Different G proteins downstream of the LPA_2 receptor are involved in regulation of the SREBP-FAS and AMPK-ACC pathways in LPA-treated cells. Our results showed that SREBP cleavage/activation lies downstream of the $G_{12/13}$-Rho pathway, and AMPK dephosphorylation/inhibition is mediated by the G_q-PLC cascade. LPA stimulated cleavage of the precursor SREBP into mature and active forms in a time-dependent manner, which was accompanied by increases in SREBP-dependent transcriptional activity and up-regulation of endogenous SREBP target genes. In addition, the effect of LPA on SREBP cleavage and activation remains sensitive to cholesterol-mediated regulation, indicating the sterol-sensing machinery involved in SREBP cleavage is not disrupted by LPA. The proteolytic cleavage of SREBP is controlled by the combined action of SCAP and INSIG proteins (64). An increase in SCAP or a decrease in INSIG proteins could lead to activation of SREBP. Because androgens and insulin have been shown to regulate expression or stability of SCAP or INSIG (65, 66), it will be of interest to determine whether LPA modulates these proteins or their ratios to activate SREBP. This possibility is consistent with the observation that SREBP cleavage occurs hours after exposure of ovarian cancer cells to LPA.

It has yet to be determined how the G_q-PLC pathway is linked to dephosphorylation and inhibition of AMPKα. Obviously, our observation does not agree with Kim *et al.* (67), who recently reported that LPA stimulated transient phosphorylation of AMPKα at Thr-172 within the first 10 min of LPA treatment in the SKOV-3 ovarian cancer cell line. In our experiments involving multiple ovarian cancer cell lines, there was little change in AMPKα phosphorylation status at the early time points. Instead, we observed a time-dependent decrease in phospho-AMPKα levels, which maximized after 12 h of incubation with LPA. The serine-threonine kinase LKB1, encoded by the Peutz-Jeghers syndrome tumor suppressor gene, is believed to be primary AMPK kinase as suggested by LKB1 knock-out studies (68–70). LKB1 possesses a nuclear localization domain and is located predominantly in the nucleus. Upon phosphorylation, LKB1 translocates to the cytoplasm where it forms an active complex with Ste20-related adaptor (STRAD) and mouse protein 25 (MO25) (71). LPA may down-regulate LKB1 activity via modulation of its phosphorylation, nuclear-cytoplasmic translocation, or association with STRAD-MO25 in the cytosol. In addition, AMPK phosphorylation could be down-regulated by inhibition of other candidate AMPK kinases such as calmodulin-dependent protein kinase kinase-β (71) or by activation of unknown AMPK phosphatase(s). A potential decrease in AMP/ATP ratio could also change the conformation of AMPK to prevent the active site (Thr-172) on the α-subunit from being exposed and phosphorylated by AMPK kinases.

Acknowledgment—Massey Cancer Center of Virginia Commonwealth University School of Medicine was recipient of National Institutes of Health Grant P30 CA16059.

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Supporting Data

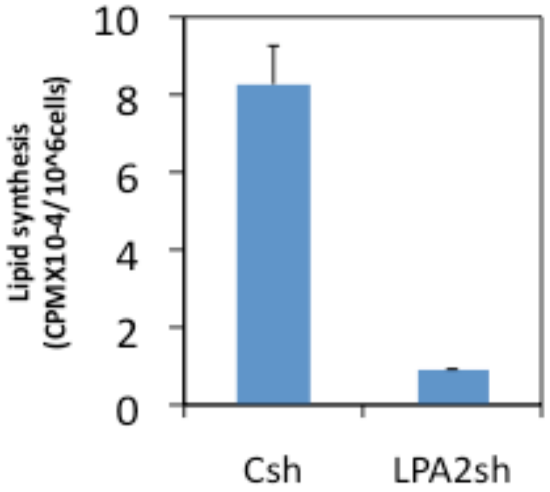
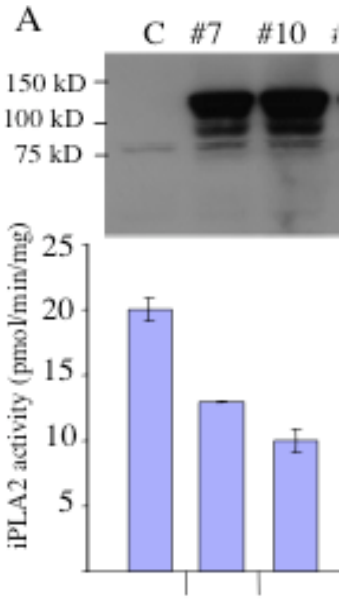


Fig. 1. Knockdown of the LPA2 receptor prevents lipogenesis of ovarian cancer cells cultured in complete medium containing 10% FBS. LPA2 in OVCA-432 cells was stably knocked down by lentivirally transduced shRNA (LPA2sh). The de novo lipogenesis was quantified as described in the attached manuscript and the lipogenic activity was compared with the cells transduced with control shRNA (Csh).



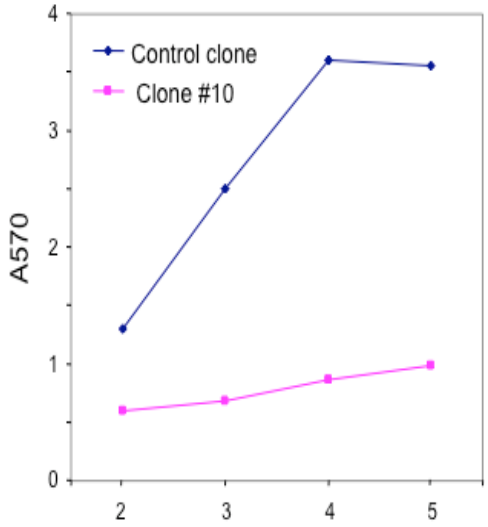


Fig. 2. Inhibition of iPLA2 activity and cell growth by stable expression of the dominant negative iPLA2b (M10iPLA2b) in SKOV-3 cells. Western blot analysis confirmed expression of the mutant in two clones (#clone #7 and #10)(*upper left*). The cellular activity was inhibited by the mutant in both clones (*lower left*). The growth curves of the control and clone #10 in serum-free conditions were determined by crystal violet staining (*right*).

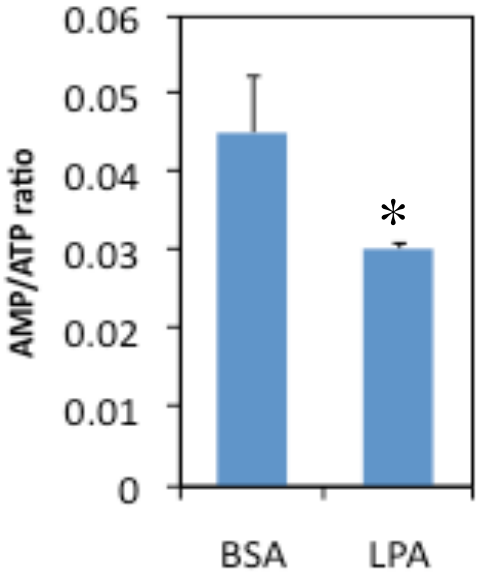


Fig. 3. Treatment of ovarian cancer cell lines with LPA increased ATP production and decreased AMP/ATP ratio. Caov3 cells were serum starved overnight prior to LPA (10 μM) treatment for 12 hours. Nucleotides were extracted and analyzed with HPLC.

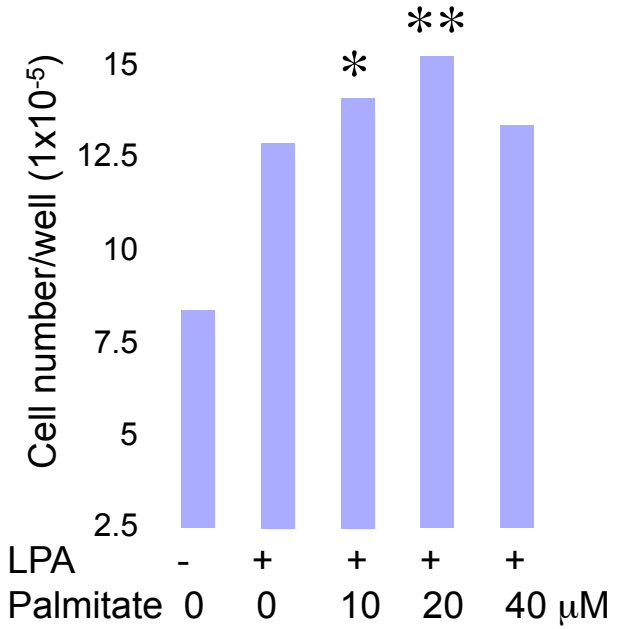


Fig. 4. Palmitate potentiates LPA-induced growth of ovarian cancer cells. Caov-3 cells were incubated with or without LPA in serum-free medium supplemented with the indicated Concentrations of palmitate. The cell numbers were determined with coulter counter after 48 hours.